A TECHNIC FOR THE INOCULATION OF BACTERIA AND OTHER SUBSTANCES INTO LIVING CELLS.*

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The method here described is an outgrowth of the technic for the isolation of single micro-organisms as described in previous papers.[1] The new feature makes possible not only the segregation of one or more micro-organisms but the injection of them as well as of measured doses of fluids into the protoplasm or vacuoles of living cells. In order to accomplish this, pipettes have to be constructed of such a fineness as to minimize the injury to the cells injected and of sufficient rigidity to pierce the cell wall. Further, an injection force has to be employed sufficient to overcome cell pressure, capillarity, and any obstruction in the pipette. The first requirement was met by modifying the method of drawing pipettes, and the second by the use of the expansion of mercury as a source of power for injection.

Each phase of the method and each part of the apparatus will be described in detail, and, for the most part, in the order that one would follow in carrying out the process.

The pipette holder (*ph*, Fig. 4) is the same as that described for the isolation method. It consists essentially of an attachment to the stage of the microscope which holds the pipette, and by means of screws allows an up-and-down and a lateral movement of the pipette (*sv* and *sl*, Fig. 4). A double pipette holder, which is described below, may be used in place of the simpler form.

Any mechanical stage may be employed which allows a considerable range in both directions. The glass box is prepared as for the isolation method. Two convenient forms have been used, the smaller 60 mm. long by 25 mm. broad by 16 mm. high and a larger one 70 mm. long by 36 mm. wide by 16 mm. high. Water is kept in the bottom of the box and, further to insure moisture, the sides are lined with wet filter paper. A number 2 cover-glass

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[1] *Jour. Infect. Dis.*, 1908, 5, p. 380; *Kansas Univ. Sci. Bull.*, 1907, 4, p. 3.

of suitable size is sealed by means of vaseline to the top of the box. This cover may be sterilized or not depending on the sort of work to be done.

The cells to be injected are placed in water on the under side of the cover, preferably toward the inner end where there is less danger of drying. An abundance of moisture may be further insured by large hanging drops of water placed under the outer end of the cover-glass, and, if necessary, by thin cloth or paper wet and so placed as partially to close the entrance of the box.

Hanging drops of the bacteria or fluids to be injected may be placed on the cover as near to the cells as possible. The broader box and cover allows more room for cells and hanging drops.

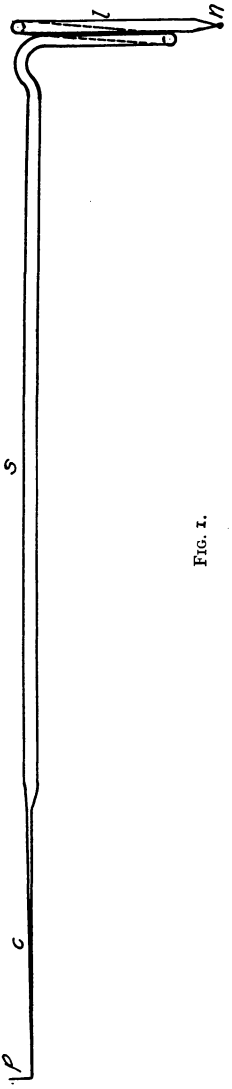

FIG. 1.

The pipette (Fig. 1) is best made from glass tubing of total diameter of about 4 mm. and wall about 0.7 mm. thick. Considerable variation in size is allowable, but one should use a somewhat thicker walled glass than is necessary for isolating pipettes. Hard or soft glass, if tough and of good quality, may be used. The advantage of the hard glass is its greater strength and the greater ease of making the fine points, while the soft glass is more easily bent and drawn out into capillaries. A piece of tubing about 35 cm. in length is bent at one end into the form shown in the illustration. The distance from the top to the bottom of the curved portion should be 4 to 5 cm. For convenience in description, the curved portion will be designated as the loop. The tip *n* is then drawn out into a moderately fine capillary and the whole tube nearly filled with mercury by

exhausting the air at the straight end, which may conveniently be done by attaching a rubber tube to this end. The point *n* is then sealed off and any air remaining above it after the sealing is worked around to the open end. The mercury should be clean and dry; it is best drawn into the tube when hot, and all precautions should be taken to insure freedom from air or water vapor. It is not essential to have the exact form of loop represented in the figure. The aim is to have the upper curves nearly on a level and to have sufficient bends to contain a considerable quantity of mercury. The greater the number of bends the more sensitive the instrument to changes in temperature, but the more unwieldy to handle. It is well to leave a point extending one or two centimeters below the lowest bend of the loop.

After sealing point *n* the straight end of the tube is drawn into a capillary about 7 cm. long and 0.3 to 0.4 mm. in diameter and with walls about 45 micromillimeters thick. The air is now expelled by vaporizing the mercury at point *s*. The capillary end is immersed in mercury, and the tube is filled with mercury as the vapor condenses. The small amount of air which enters when the tube cools to room temperature may conveniently be expelled by heating the loop in the flame. The capillary tip is now immersed in mercury and the loop cooled in ice water. The tube should be as free from air as possible, though a very small bubble of air does not prevent its successful use. If the pipettes are not to be used for some hours or days the capillary end may be sealed.

In making the fine capillary points for piercing cells the first step is the withdrawal of the mercury from the end of the capillary portion. This is done by immersing the looped end in ice water. The pipette is then held in the left hand, the tip of the capillary grasped by fine-pointed forceps held in the right hand, and the portion of the capillary left free by the contraction of the mercury held over, not in, a very small and narrow flame. A very convenient burner for producing a flame of this sort is made by bending a piece of hard glass tubing nearly at right angles, pinching one end into a very narrow slit, and attaching the other end to rubber tube connected with the gas supply and provided with a clamp to regulate the size of the flame. (See Fig. 2.) The

smallest flame that can be kept alive, if possible about 2 mm. high, is most serviceable. Natural gas for the microburner may be improved by causing it to pass through alcohol or benzine.

When the glass begins to soften over the flame one should pull with the forceps directly away from the other hand. The success

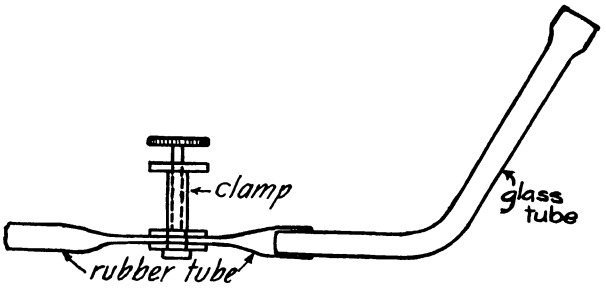

FIG. 2.

of the process depends on the timing and strength of the pull, a matter difficult to describe since much depends on the feeling. If the capillary separates with little or no feeling of resistance, the point is likely to be too long and flexible. If separation is accompanied by a snap, the point is usually too blunt. An intermediate resistance, felt as a slight tug just as the glass separates, indicates the right amount of heating and pull. After a little experience one can be sure that he has the right sort of a point by the feeling alone; but it is better to inspect the point under the low power of the microscope before proceeding farther.

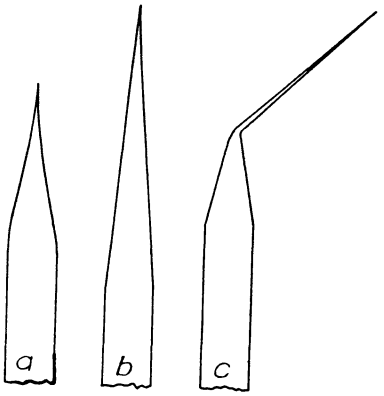

FIG. 3.

The best sort of point is shown in *a* of Fig. 3. This form combines fineness of point with rigidity and a comparatively large lumen near the tip. The end is sealed at this stage of the process. The form represented by *b* (Fig. 3) may be used, but it is more

likely to become obstructed and does not allow the regulation of the dose as well as does the form *a*. After a good point is made, the end of the capillary should be turned up at right angles as shown in Fig. 1, *p*. This is done by holding the capillary just near enough to a small flame to soften the glass while the end is lifted by the tip of the forceps. In order that the heated air in the capillary may not by expansion burst through the softened glass, a negative pressure is created in the capillary by immersing the loop in ice water just before bending the tip.

If the pipette is to be used with the mercury under considerable pressure at room temperature, the same process as described above is followed except that, just before making the point, the end of the capillary is immersed in mercury, first expelling air, if any is present, by gently heating the loop. The loop is now immersed in ice-cold water and as much mercury drawn in as possible. Then the tip of the capillary is exposed to air and the mercury is drawn from the end by immersing the loop still farther in cold water, or by cooling the portion of the tube above the loop. The pipette point is now made as described above.

The pipette is now placed in the holder, the tip brought into the center of the low-power field, and the apparatus for regulating the pressure adjusted. Two methods have been used for regulating the pressure. In the one method, the pipette is so made that the mercury in it is under considerable pressure at room temperature, as described in the preceding paragraph. In the other method, the mercury is under no pressure or a very slight negative pressure at room temperature. It will be more convenient to describe the higher-pressure method first.

The apparatus (see Fig. 4) consists of a brass tube *t* to the lower part of which is attached a brass cup *c*. This tube is held in the sleeve *s* which is attached to the arm of a simple microscope provided with a rack and pinion *r*. By means of the rack and pinion the sleeve and tube may be raised and lowered through about 5 cm. To the upper part of the sleeve a stiff wire is attached holding in a ring at its free end the bag *b* made of thin rubber tissue. All parts are adjustable. The bag may be raised, lowered, or moved laterally, the tube may be raised or lowered in the sleeve,

and a joint at *j* allows bag and cup to be swung aside together. A pan is placed beneath the cup to receive and carry away waste water.

The loop of the pipette is placed between the bag and the cup and these are so adjusted that when the sleeve is racked down to its fullest extent the upper part of the loop of the pipette will be in close contact with the bottom of the bag, and when it is racked

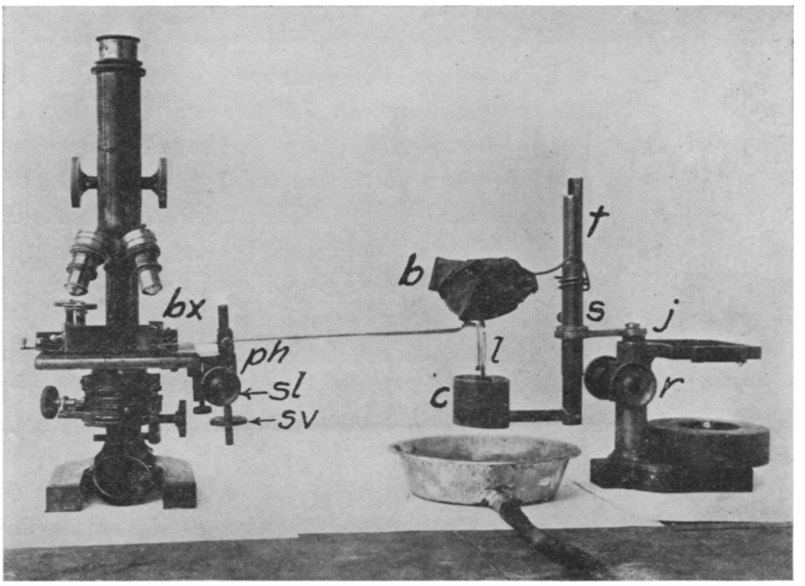

FIG. 4.

up the loop will be free from the bag and a large part of it immersed in the cup. The cup is now filled with ice-cold water and racked up so that the loop is fully immersed in it, and the point of the pipette is adjusted in the center of the high-power field. In doing this one must be careful not to break the delicate point on the cover-glass. One may avoid this by selecting under a low power a field near the margin of a hanging drop of water, and bringing the point of the pipette into this drop without touching it to the glass. The tip may then be found with the high power. If the oil immersion is to be used it is convenient to choose some conspicuous

object in a droplet of water, as a particle of foreign matter, and arrange the point of the pipette just below this object under the low power. The tip may be lowered slightly and the object found with the oil immersion. If the cover-glass has not been moved the positions of the particle and of the tip of the pipette will correspond. One has to be especially cautious in adjusting the oil immersion. A slight pressure exerted on the cover-glass in focusing may bend it down far enough to break the pipette, should the tip be left too near the glass.

When the point is found under the high power, the end, still sealed, is brought into contact with the cover-glass. Then by movement with the mechanical stage the point is scratched on the cover until the tip is broken off and the lumen of the tube opened. By varying the pressure on the cover-glass the amount broken off and the size of the opening made can be regulated. If bacteria are to be handled with the pipette a larger opening is needed than is necessary for liquids. If desired, a tube having a diameter of a micromillimeter may be made.

Next the air above the mercury must be expelled. If the mercury in the pipette is under great pressure at room temperature all that is necessary is to lower the brass cup and expose the loops to room temperature. When all of the air is driven out and the mercury appears at the tip of the pipette, pressure may be stopped by lifting the cup of ice water around the loop. If the room temperature is insufficient or too low, the rubber bag into which hot water has been poured may be racked down until its bottom is in contact with the upper part of the loop. A vessel supplied with a Bunsen burner or electric heating coil and placed somewhat above the laboratory table is a convenient source of hot water. The water may be brought to the bag through a rubber tube provided with a pinch cock.

In order to fill the pipette, the point filled with mercury to the tip is brought into a hanging drop of the liquid to be injected and the ice water racked up so as to surround the loop. The contraction of the mercury brings about a negative pressure and draws the fluid into the pipette. When a sufficient dose has been drawn in the water is partially lowered from the loop until the inflowing

ceases. The point is then lowered a short distance below the cover, and by means of the mechanical stage the cell to be injected is brought immediately over the point. The point is raised, the cell wall pierced, and the point brought to the vacuole or to any part of the cell desired. The loop is then further exposed to room temperature, and, if necessary, the bag containing hot water is lowered into contact with it. As soon as the dose is injected the cold-water cup is racked up and the pressure stopped. The pipette may be withdrawn or the action on the cell of the injected substance may be observed while the point is still in.

The second method is somewhat simpler. Adjustments are made as in the first process except that the use of warm water in the rubber bag is dispensed with, and warm water is brought into the brass cup instead. Since the mercury in the pipette is under no pressure at room temperature, warm water is brought around the loop in order to expel the air. The pipette is charged by bringing the point into the liquid or emulsion to be injected, lowering the cup, and exposing the loop to room temperature. If there is too much pressure in the pipette for convenient filling some of the mercury may be expelled by immersing the loop in warm water or the loop may be cooled by lifting a beaker of cold water around it. After being charged the point is introduced into the cell and the contents expelled by immersing the loop in warm water in the brass cup. The loop may be only partially immersed, or the water in the cup kept only slightly above room temperature if but light pressure is needed. If greater pressure is demanded, warmer water may be introduced into the cup. As a rule one should use the lowest pressure necessary for expelling the contents of the pipette.

The advantages of the second method are its simplicity and the ease with which one may obtain any desired pressure by regulating the temperature and the amount of the loop immersed. It has the disadvantage that air temperature may not be low enough to bring about a sufficiently sudden contraction of the mercury, and the injection process may not be stopped quickly enough to prevent an overdose or the injection of mercury into the cell. This disadvantage may be lessened by using the rubber bag kept

full of ice water. On the whole, the first-described process has thus far proved more convenient and reliable. It affords at least one constant, the temperature of ice water, and the room temperature is sufficiently constant for practical purposes. It is furthermore of greater importance to be able to stop the injection process quickly than to be able to apply pressure rapidly.

It is evident that an apparatus of this sort, which consists simply of a device by which heat or cold can readily be applied to a loop of glass containing mercury, may be modified in several ways. Some of these modifications have been tried, others are now being experimented on. The rubber bag may be partially filled with oil and a small electric light bulb or other electric heating device may be so arranged that the oil will be kept hot; or the bag may be dispensed with and radiated heat from some source substituted. Any electrical heating device may be arranged for keeping the water in the brass cup at any desired temperature, or for the cup a ring suitable for holding a beaker of any depth is a convenient substitute. The combination of cold water below and warm water above has thus far seemed the most feasible plan for meeting the main requirement—the gradual application of heat and the more sudden application of cold. Water may be substituted for mercury in the pipette and has the advantage of greater lightness, but the greater specific heat and conductivity of mercury makes it preferable.

In order that the dosage may be regulated and that mercury may not be driven into the cell the tip of the pipette should be unobstructed when it pierces the cell wall. Substances may successfully be injected in spite of a clogged tip, but so much force is often required to overcome the obstacle that ejection takes place with a rush and mercury is likely to follow the dose. Further, both for the regulation of the dosage and the prevention of clogging, pressure in the pipette should be nearly in equilibrium at the time of the entrance of the point within the cell. Positive pressure may not be a disadvantage if one does not care to avoid ejecting some of the contents of the pipette outside of the cell, but a too strong negative pressure causes the pipette to take up liquid surrounding the cell and increases the danger of clogging when the point enters

the cell. A clogged pipette may often be opened by gently scratching it on the surface of the cover-glass. Sometimes it is necessary to enlarge the opening somewhat.

If a volatile liquid is to be injected, or if large or repeated doses of the same substance are to be used, one may fill part of the capillary with the substance to be injected before making the injecting point, but in most cases one had better regulate the dosage by filling from the point. If very small doses are to be injected, one may keep the top of the mercury column in view after charging. The cell is pierced, pressure applied, and the rising of the mercury column to the tip shows that the dose has been injected. Focusing on the mercury column may be facilitated by piercing the cell obliquely instead of from directly below. With the use of larger doses the top of the mercury column is usually below the reach of the lens. Here one can focus as far down as possible in the pipette after its introduction into the cell and stop the pressure on the appearance of the mercury column. If pipettes of the form represented in Fig. 3, *a*, are used, much more force is required to expel the mercury than to bring it to the tip, so that one has time to stop the pressure after the appearance of the mercury. If is it desired to remove cell contents the retreat of the mercury column indicates that the contents are being drawn into the pipette.

Small doses may be measured by estimating the cubic contents of the pipette between the top of the mercury column and the tip. Larger ones may be estimated by expelling the dose on the cover-glass and measuring the droplet expelled. The liquid is then drawn into the pipette again. If a definite number of bacteria are to be inoculated, they may first be isolated in a droplet of fluid and the whole droplet inoculated.

If the substance to be inoculated forms a precipitate with the mercury, a quantity of water or of some indifferent oil, sufficient to separate the substance to be injected from the mercury, may first be drawn into the pipette.

One should have the cell to be injected well located before filling the pipette and as near to the filling place as possible, so that little time will be lost between charging and injecting. This is the

more necessary where it is advisable to keep the pressure in the pipette in equilibrium after filling. Lines may be drawn on the cover-glass to serve as guides, the cell may be located by means of the vernier on the mechanical stage, or the droplet of substance to be injected may be placed just in line with the cell so that only one movement of the mechanical stage is necessary in passing from the one to the other.

The penetration of the plant cells thus far experimented on is easy if the tip is made fine enough and if it contracts abruptly enough so that the pipette has the necessary stiffness. If points are too pliable, there may be difficulty in penetrating the cell; if too blunt there is danger of tearing the cell wall. The loss of some of the injected fluid or of cell contents on withdrawal of the pipette cannot always be avoided, but it may be minimized and in some cases entirely prevented by withdrawing the tip very slowly so that the protoplasm has time to form a plug over the opening. Further, if the tip is brought just within the cell it may be introduced with less injury to the cell and withdrawn with less loss of liquid. The smaller the tip the less injury to the cell and the greater the ease of withdrawal.

In the multinuclear cells of the fungus group Saprolegniaceae and of Nitella and Vaucheria among the algae the cell wall has been pierced, different substances injected, and the pipette withdrawn with little or no apparent injury to the cells, as judged by the movements of the protoplasm and the subsequent behavior of the cell. With some mononuclear algal cells, as Spirogyra, the cell appears to be more sensitive to injury. Few animals have thus far been experimented on with the injection apparatus. Mercury has been injected into rotifers and substances into Paramecium, but the technic for inoculating animals has thus far not been fully developed.

Any objectives from the lower powers to the oil immersion may be used. The oil immersion should have sufficient depth of focus to reach the bottom of the cell. The cells to be injected should be in contact with the cover-glass or as near to it as possible.

In making a new capillary point the pipette may be removed from the holder, the old point broken off, and a new one made

from the same capillary. When the capillary is used up a new one may be made from the end of the straight portion of the pipette, and the process continued until all is used back to the loop.

In certain kinds of work it is of advantage to use two pipettes simultaneously. This makes necessary the use of a special holder for two pipettes, a modification described in previous papers. The holder is so arranged that each pipette has its own movements, up and down, in and out, lateral and rotary with the axis at the point of attachment in the holder, and the two pipettes move together laterally besides. With this apparatus one may inject two different substances into the same cell at the same time, or by varying the pressure inject with one pipette and withdraw with the other. One pipette may be used simply as a probe or dissecting instrument, or it may be attached to a rubber tube and used as an isolating pipette, while the other is arranged for injection. The injection apparatus may be dispensed with, the tips of the pipettes left closed or made from a solid rod, and used either singly or in conjunction as needles or dissecting instruments. One or both of the pipettes may at once be converted into isolating pipettes, or through them one may treat the dissecting parts with stains, fixatives, or other reagents. An improved type of the double pipette holder is now being constructed under the direction of Dr. F. Hecker of this laboratory.

With a single point the writer has successfully divided amebae and also removed the nuclei of amebae, with little loss of protoplasm or apparent immediate injury to the organisms. The division of amebae has been accomplished with the straight point and with one bent at a considerable angle (Fig. 3, *c*). With two points spores of fungous parasites have been removed from their host, and sporangia of fungi have been dissected. In one experiment a rotifer has been held by the blunted point of one pipette while mercury was injected into the body with the other pipette. Points may be made fine almost to invisibility with sufficient stiffness for piercing the wall or even the nuclear membranes of cells.

It has seemed to the writer that this technic in its different forms may assist in the solution of various problems in the biology

of microscopical plants and animals. Experiments are now being carried on in this laboratory on the infection of cells through the injection of various micro-organisms and on the effect on cells of different chemical substances inoculated directly into the protoplasm or vacuoles. The introduction of foods, poisons, stains, and fixatives is made possible and cells may be probed or dissected under high powers, methods which may be of use in the study of the structure, chemistry, and physiology of cells. Finally, materials may be withdrawn from one cell and injected into another, and it is possible that investigations on fertilization and heredity may be extended by this technic.

NOTE.—In the process of isolation of micro-organisms the use of points such as those illustrated in Fig. 3, *a*, will be found an improvement on those described in earlier papers. The pipette is fixed in the holder and focused under the higher powers while the tip is still closed. The point is brought into a hanging drop of broth or other fluid to be used and the tip opened by gently scratching the cover-glass, as described for the inoculation pipettes. An opening of the size suited to the micro-organisms to be manipulated is made, the pipette filled from the drop, and the process carried on in the usual way. Further it will be found of advantage to clamp the pipette holder on a plate screwed on to the stage of the microscope instead of to the stage itself. (See Fig. 5.) This brings the pipettes nearer to the level of the stage and allows more working room for the mechanical stage. The longer isolation box described above will be found of advantage in allowing more room and in keeping the hanging drops under better conditions of moisture; and the broader form of box and cover will be found useful in some forms of isolation.

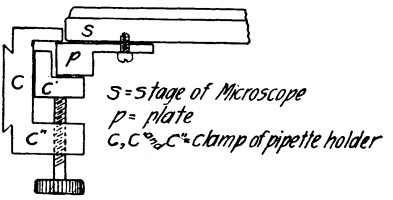

FIG. 5.